REVIEW ARTICLE

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An Overview of Microorganisms' Contribution and Performance in Alcohol Fermentation Processing a Variety of Substrates

Poonam Singh Nigam*

School of Biomedical Sciences, Faculty of Life & Health Sciences, University of Ulster, Coleraine, Northern Ireland, UK

Abstract:

Background: There are some chapters written on microbiology of alcohol-making, where grapes and certain regional and seasonal fruits have been used to produce wines or specialized fruit beverages. This overview deals with the types of microorganisms involved in alcohol (ethanol) fermentation using various types of substrates, such as agricultural, industrial by-products and residual substrates, and seasonal and regional fruits. The type of micro-flora controls the process of alcohol production, which could be pure *saccharomyces* yeasts, exo- and endogenous non-*saccharomyces* yeasts, and bacteria depending on the type of substrate used. Review incorporates information on some processes which have been recently developed for the use of immobilized microbial-cells in fermentation, simultaneous alcoholic and malolactic wine fermentation, simultaneous saccharification and fermentation and co-culture system to process different types of substrates, available globally as by-products or residues.

Methods: A search of bibliographic references for peer-reviewed research literature was undertaken focussing on two aspects – first on the types of substrate have been used for ethanol production, and second on specific types of microorganisms employed to ferment these substrates.

Results: Forty-seven most relevant papers were included in the review of this topic. Information gathered on both aspects as described in methods, has been structured in suitable sections of review.

Conclusion: The findings of this review confirm the importance of selection of a suitable microorganism, *Saccharomyces*, or non-*Saccharomyces* is important in ethanol fermentation, particularly depending on the type of substrate being used for the fermentation, a traditional fruit or agro-industrial waste. Certain modifications and adaptations could be incorporated in the fermentation system using these microorganisms as biocatalyst, to improve their performances and the overall productivity and the quality of the product.

Keywords: Yeast, *Saccharomyces cerevisiae*, molasses, immobilization, *Lactobacillus*, ethanol.

1. INTRODUCTION

A variety of substrates have been utilised for alcohol production, such as agricultural and industrial by-products including: sugarcane molasses from sugar industry; whey and lactose containing effluents from cheese production; agricultural residues such as grains, starch, sugarcane bagasse, sugar beet pulp; and regionally produced fruits. Therefore, a specific type of microorganism has to be employed to utilise the sugar available in and from these substrates and to bio-convert it into ethanol. The alcohol produced from different substrates may have potable and non-potable applications. The alcohol made from a variety of fruits, other than grapes, have utility as fruit-wines [1-3],

whereas alcohol produced from a variety of agricultural residues and biomass may have other applications as bio-fuels [4, 5].

2. MICROORGANISMS CONTRIBUTION IN FERMENTATION OF VARIOUS SUBSTRATES

The microorganisms involved in the process of alcohol synthesis could be endogenous and exogenous, depending on types of substrate being used in fermentation. The exogenous organism is the purposely-selected species of a microbial-culture, whereas the endogenous cultures are contributed by substrate itself, such as from the substrate-surfaces, particularly in fermentations using grapes, berries and other fruits. The quality of final product is controlled by the source of organism, but it also depends on the contributions made by type of microorganisms present in fermentation system, yeast/s alone or bacteria and yeast together.

*Address correspondence to this author at the School of Biomedical Sciences, Faculty of Life & Health Sciences, University of Ulster, Coleraine, Northern Ireland, UK; E-mail: p.singh@ulster.ac.uk

2.1. Alcohol Fermentation Using Molasses from Sugar Industry

Sugar industry produces molasses as a large amount of by-product, which is rich in sugar content. The main utilisation of this valuable by-product has been established for the production of alcohol in optimised fermentation processes using suitable strains of yeast [4, 5]. Farrell *et al* [6] studied and optimised a process for increased alcohol fermentation efficiency using sugarcane molasses, by a process of pre-treatment with Amberlite and non-living biomass. A continuous alcohol fermentation process using molasses was optimised using *Saccharomyces cerevisiae* cells immobilised on alginate matrix [7]. A column reactor of immobilised yeasts was used for continuous alcohol production using molasses in a continuous process [8].

A thermotolerant yeast strain of *Kluyveromyces marxianus* has been employed to ferment molasses at higher temperature in warm climatic conditions, and the process was further improved by the use of the yeast cells immobilised in alginate matrix [9]. Molasses fermentation has also been tried using thermotolerant strains of yeast at industrial scale [10], and a selected strain of thermotolerant yeast *K. marxianus* has been used in Indian distillery by Singh *et al* [11] to avoid water cooling of fermenters in hot months of summer, which was otherwise required with the use of conventional distillers' yeast *Saccharomyces cerevisiae*, which produces optimum alcohol at 37 °C.

2.2. Alcohol Fermentation Using whey and Lactose from Cheese Industry

Whey is produced as a by-product in large volumes in cheese industry. The main sugar present in whey is lactose, which could be utilised for alcohol fermentation. Kourkoutas *et al* [12, 13] have studied in detail the fermentation of whey for alcohol production at high temperature using thermotolerant yeast strain. A cheaper support for the immobilisation of yeast cells to ferment whey has been used in form of delignified cellulosic material [14]. Lactose sugar in effluent of cheese factory were fermented to produce ethanol, process has been optimised using thermotolerant yeast strain *K. marxianus* [15-17]. This yeast performed differently and to a better efficiency, when used for fermentation in immobilised forms in alginate matrix [15] and in magnetically responsive matrices [16]. Brady *et al* [17] found that Mn⁺ in fermentation system affects the activity of yeast cells and thus the overall alcohol production from lactose can be manipulated for better yields by the use of yeasts in suitable forms.

2.3. Alcohol Fermentation Using Agricultural Substrates

Several residual substrates from agriculture such as spent barley grains from distilleries and breweries are good source of carbohydrates for fermentation. Sorghum and tapioca have been found as good source of sugars, studies have been performed for the enzymatic hydrolysis of these cheaper agricultural materials to produce fermentable sugars suitable for alcohol production [18,19]. Agricultural residues and by-products have been used for alcohol production using several types of microorganisms in specially designed submerged fermentation systems in batch or fed-batch process [20].

2.4. Alcohol Fermentation Using Apple-Must

The apple fermentations may use the wild micro-flora present on the surface of fruits, and other yeasts in wineries which are indigenous. The product in such fermentation is traditional cider produced by the organisms, which are naturally present on apple. Different countries have their own cider making process, such as Brazilian cider is produced using specific yeasts such as, *Brettanomyces* sp., *Hanseniaspora valbyensis*, *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* var. *uvarum*. Cider produced in France uses traditional complex natural micro-flora, which are requisite for partial slow fermentation of sugars to retain some residual sugars.

The traditional cider fermentation from apples does not employ the inoculation of specific yeast strains, where the apple juice is initially fermented by dominating non-*Saccharomyces* species contributing specific flavours [21, 22]. But the initial natural micro-flora does not exist longer, as the alcohol concentration increases in the fermentation, and a later stage *Saccharomyces cerevisiae* var. *uvarum* takes over producing wine-flavour. Traditional ciders generally undergo malo-lactic fermentation, which is known as secondary fermentation or maturation. This process is controlled by acid-tolerant bacteria *Lactobacillus* spp. and *Leuconostoc* spp [1-3].

Herrero *et al* [23] controlled malolactic fermentation in cider using oenococcus in its immobilised form rather using free cells. Currently many cider producers use naturally selected *Saccharomyces* strains for the reason that the fermentation can be easily standardised [24]. The strains used for apple fermentation could be different in different countries. In making of Spanish cider two different strains of *Saccharomyces bayanus* and *cerevisiae* were employed. The cider produced by the use of *Saccharomyces bayanus* contained two polypeptides of high molecular weight and two of medium molecular weight. The ciders made by the use of *Saccharomyces cerevisiae* contained two polypeptides of medium molecular weight and two of low molecular weight. These polypeptides control the high and lower foam-stability of ciders, respectively. The sparkling ciders have been produced using Champenois method using *Saccharomyces bayanus* C6 and *Saccharomyces cerevisiae* strains.

The conventional cider fermentation can be performed as controlled, which is possible using sequential inoculation of *Saccharomyces cerevisiae* and *Leuconostoc oenos*. The storage of cider can face certain disorders, like Cider-sickness due to the development of a bacteria *Zymomonas anaerobia*. This organism produces ethanol and acetaldehyde compounds, which together with phenolic compounds cause turbidity a colloidal cloudiness that brings a change in flavour. The cells of *S. bayanus* and *Leuconostoc oenos* immobilised in alginate matrix were used in continuous fermentation of apple juice to improve technological and sensory qualities of cider.

Continuous cider fermentation was achieved using co-immobilised yeasts and bacteria [25]. The continuous process reduces the retention time and therefore reduced fermentation time of apple juice allowed better control on flavour formation, which is not possible in a batch

fermentation system retaining apple juice for longer periods. The quality of cider as soft or dry can be controlled by adjusting the residence time of feeding apple juice in fermenter.

2.5. Alcohol Fermentation using Juicy Fruits

Certain juicy fruits are excellent material suitable for a specific alcohol-product as their pulp contain a range of sugars and organic acids, which produce volatile and phenolic compounds [1-3]. The ingredients/composition of a specific fruit like mango, peach, plum, pear, kiwi-fruit, strawberry, or citrus-fruits etc used for alcoholic fermentation, contribute to the desired sensory quality of final product, and accordingly the type of fruit is selected for a specific product such as apples for making cider; peaches, blueberry, cherry and strawberry for specialised alcoholic beverages [3]. Commercial preparation of wine-yeasts are available in the form of active dry yeasts (ADY), which are used in modern wineries to prepare specialised wines for commercial production [2,3].

The fruit juices are inoculated with standard yeast to ferment the sugars to alcohol. Some other microorganisms other than yeasts also play an important and additional roles in process. All microorganisms together play an important role in developing complex aroma and flavour. Their microbial activities contribute in the development of a desirable quality of product, producing the mixture of different alcohols and compound in the fermented broth to add the distinct aroma and flavour.

The microbiology of the wine making process incorporates the significance in infection-proof-completion of the appropriate fermentation, and then strictly managing the post-fermentation process for proper maturation and prevention of the taste-spoilage. The several issues related to wine making have been the focus of research, including the natural indigenous and exogenous micro-flora, progression of fruit micro-flora, the activities of *Saccharomyces* and non-*Saccharomyces* yeasts in fermentation, the role of yeasts and certain bacteria, potential of pure and spontaneous yeasts cultures, relative merits of spontaneous versus induced fermentations, etc.

Certain fruits are found to produce better quality fruit-beverages due to maximised colour and flavour e.g. plums [26, 27], papaya [28], mango [29]. The non-infected fruits may contain an indigenous microbial population of 10^3 to 10^5 cfu per gm of fruits, which have been found of consisting a range of microorganisms, including- yeasts *S. cerevisiae*; apiculated yeasts - *Kloeckera* and *Hanseniaspora*, and a lower occurrence of *Candida*, *Cryptococcus*, *Pichia* and *Kluyveromyces*. Bacteria as part of indigenous microbial population are mainly Lactic acid bacteria, including several species of *Lactobacillus*, such as *plantarum*, *brevis*, *cellobiosis*, *buchneri*, *casei*, *mesenteroids*, and *trichodes*. Other bacterial strains found on fruits are *Leuconostoc oenos*, few species of *Pediococcus* including *pentosaceus*, *damnosus*, and *parvulus*. *Gluconobacter*, and *Acetobacter* have also been detected. The indigenous fungal strains on fruits are mainly *Botrytis*, *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Ucinula Alternari* and *Cladosporium* [2].

S. cerevisiae eventually dominates most of the wine fermentations. Consequently, it is widely recognized as the

principal wine yeast and is the species that has been commercialized as starter cultures. Oliveira *et al* [30] used both forms of yeasts, as free cells and immobilised cells, in fruit-beverage production using cagaita fruit. However, wine fermentations induced by inoculation with selected strains of *S. cerevisiae* are not devoid of contributions from the natural yeast flora. In inoculated fermentations, *S. cerevisiae* is usually added to achieve a population of about 10^5 to 10^6 cells per ml in fruits-must. The indigenous *S. cerevisiae* grape flora often constitutes an additional 10^4 to 10^5 cells per ml.

A study using mitochondrial DNA restriction analysis has shown that although the inoculated strains may soon become dominant, other *S. cerevisiae* may remain active for several days before being suppressed. Thus, indigenous strains of *S. cerevisiae* may play a significant sensory role during the early stages of fermentation. The use of a selected strain allows an early and healthy fermentation start and reduces the occurrence of fermentation problem. For the commercial production of wine, strains of *S. cerevisiae* are available. The information on important properties and trade names of yeast strains and their fermentation characteristics are available on www.DSM-oenology.com.

3. PROPERTIES OF PRODUCT CONTROLLED BY MICROORGANISMS

The organo-leptic properties of final product depend on the activities of micro-flora. The growth and metabolic activities of a variety of endogenous and exogenous microorganisms contribute to oenological properties, due to the reason their involvement occurs during the whole process, including fermentation and post-fermentation stages [1, 2].

Climatic conditions during the growth of grapes considerably affect the occurrence of endogenous non-saccharomyces yeast micro-flora in different countries [3]. At the initial stage of fermentation, the population of *Saccharomyces* is very insignificant; several non-*Saccharomyces* yeasts such as *Aureobasidium pullulans*, *Candida stellata*, *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Kloeckera javanica*, *Metschnikowia pulcherrima*, *Pichia anomala* and *H. uvarum* are found dominant in the early stages of fermentation in California and mid-Europe.

Following properties of alcoholic product develop in due course of process due to microbial activities:

Easty taints due to higher concentration of ethyl acetate and methyl butyl acetate produced by *Hanseniaspora* species; Film formation on wine surfaces, and severe flavour taints by *Candida*, *Metschnikowia*, and *Pichia*; Turbidity, haze, volatile acidity, phenolic taints in stored wines by *Brettanomyces* spp and *Schizosaccharomyces pombe*; Mannitol taints by *Lactobacillus brevis*; increase in the acidity due to production of lactic and acetic acids by *Lactobacillus* and *Leuconostoc* spp; Ropiness by *Pediococcus cerevisiae*; Production of diacetyl by *Lactobacillus*, and *Pediococcus* spp.; Production of bitterness due to acrolein taint by *Leuconostoc* spp.; Breakdown of tartaric acid and miscellaneous off-flavours by *Lactobacillus* spp.; Acidic and estery taints caused by the aerobic metabolism of *Gluconobacter oxydans* and

Acetobacter pasteurianus; Vinegary taints due to production of acetic acid in higher concentrations of 1.2 g/l by *Acetobacter aceti*; An increase in volatile and total acidity by various species of *Bacillus*; Production of corky and earthy taints by *Actinomyces* spp., and *Streptomyces* spp.; Earthy and corky taints by fungal growth in wooden barrels and in bottle corks.

4. MICROBIAL PERFORMANCE CONTROLLED BY TEMPERATURE

The concentration of alcohol produced depends on the initial sugar concentration present in system and the variation in fermentation temperature may have a phenomenal effect on alcohol yield. The patterns of microbial succession and their dominance are greatly influenced by the temperature maintained in system. Temperature has an impact on development of yeast and its fermentation kinetics [4,31,32]. Temperature can have an important role in the existence of indigenous yeast and yeasts ecology. A cryotolerant *Saccharomyces cerevisiae* promoted fermentation of maltose containing medium at extremely low temperatures [33]. Temperature profoundly affects yeasts' respiratory and fermentation intensity, which is a measurement of oxygen consumed or carbon dioxide released mm³ per gm of dry yeast per hour. The optimal temperature of ethanol fermentation is different for each type of yeast strain. At 25 °C, *S. exiguus* and *Zygosaccharomyces bailii* may predominate in system, but at lower temperature 10 °C, though *S. cerevisiae* is dominant, but *K. apiculata*, *Candida stellata* and *C. krusei* may also develop in large numbers.

Different strains become more or less adapted to different temperatures in the range of 10 to 30 °C. As a result, the growth rate varies for each strain with the change in fermentation temperature; for example in case of *Kloeckera apiculata* and *S. cerevisiae* the fermentation intensity have been noticed to increase by a factor of two for the increase in temperature of every 10 °C, reaching to maximum at 35 °C, and then start decreasing above 35 °C. The fermentation of fruits sugars occurs at twice the rate at 30 °C as at 20 °C. The alcohol producing yeast have capability of transforming 10% more sugar in the same elapsed fermentation time for each temperature increase of 1°C.

In addition to the influence on yeast activity, temperature is also responsible for the speed of fermentation and its limits. Between 15 and 35 °C, the duration of the latent phase and delay before the initiation of fermentation become shorter with the increase in temperature. A fruit-must with a limited sugar concentration and less than 200 g per litre, takes several weeks to ferment at a lower temperature of 10 °C, 15 days at 15 °C, while the duration of fermentation becomes very short by 3 to 4 days at 30 °C. In case of musts having higher sugar concentrations available for fermentation, the fermentation becomes more limited with the increase in temperature.

At higher than optimal temperature, fermentation can stop leaving sugars unfermented, though thermotolerant yeasts have been isolated growing at 52 °C [31] and producing ethanol at 45 °C and 50 °C, even at large scale [10].

5. BACTERIAL CONTRIBUTION IN ALCOHOLIC FERMENTATION

Lactic acid bacteria (LAB) are next generation starter cultures for malo-lactic fermentation [34]. LAB are characterized by their unique properties and they have capability to produce large amounts of lactic acid. LAB have been employed for malo-lactic fermentation by Genisheva *et al* [35] in their immobilized form. Depending on the genus or species, lactic acid bacteria perform two types of fermentations: 1. Homo-fermentation - sugars are fermented solely to lactic acid; 2. Hetero-fermentation - the products could be ethanol, carbon dioxide and lactic acid. Homo-fermentation yields two ATPs per glucose which is similar to yeast fermentation, whereas only one ATP is produced in hetero-fermentation. One of the hetero-fermentative and most beneficial bacteria is *Leuconostoc oenos*. This is also the most frequently occurring species of lactic acid bacteria inducing malolactic fermentation in wines of low pH (3.5). Spoilage forms of bacteria are generally members of the genera *Lactobacillus* and *Pediococcus*. Out of these two genera, *Lactobacillus* contains species with ability of homo- and hetero-fermentations, while *Pediococcus* is strictly homo-fermentative.

All lactic acid bacteria growing in wine assimilate acetaldehyde and other carbonyl compounds. The metabolism of carbonyl compounds may retard malolactic fermentation by liberating sulphur dioxide bound to carbonyl compounds. Although lactic acid bacteria grow under acidic conditions, their growth is poor in must and wine. Species of *Lactobacillus* and *Pediococcus* commonly cease growing below pH 3.5. Primary malolactic bacterium *Leuconostoc oenos* is unable to grow below pH 3.0. It grows optimally within a pH range of 4.5 to 5.5; thus, the major benefit of malolactic fermentation for the bacteria may be acid reduction. By metabolising a dicarboxylic acid such as malic acid, to a monocarboxylic lactic acid, acidity is reduced and pH is increased. The increased pH provides favourable conditions for bacterial propagation. Malolactic fermentation in fruit wines may not be desired since it can cause significant loss of acidity, may produce undesirable changes in flavour and result in high wine pH.

6. PERFORMANCE OF MICROBES IN IMMOBILIZED FORM

Microorganisms in their immobilised form [36] have been tested for their performance for a better yield as well as for a continuous alcohol production [15-17, 37]. Love *et al* [38, 39] employed high temperature performing yeast by immobilizing on two types of supports. *Lactobacillus casei* cells have been immobilized on delignified cellulosic material (DCM) and used for malolactic fermentation (MLF) of wine [40]. In first stage of process the wine was produced employing yeast cells immobilized on DCM at 20 °C, and in second stage after the completion of alcoholic fermentation, MLF was started using immobilized *L. casei* cells at 27 °C.

The immobilised microbial cells have been specially designed for the purpose of recycling as economical biocatalysts, a total of 11 (eleven) repeated batches of alcoholic and subsequent MLF could be performed within a continuous period of one month. In such experiments after

few repeats of batches, the MLF activity of the immobilized biocatalyst was reduced. Wine samples were analysed by GC-MS before and after MLF, revealing some qualitative differences. Malic acid degradation was reduced from 80 to 2%, pH was reduced by 0.5–0.1 unit, acetic acid concentrations were slightly reduced or remained stable (0.002 g/L), the higher alcohols 1-propanol, isobutyl alcohol, and amyl alcohol were decreased by 84, 23, and 11%, respectively, and ethyl acetate concentration was increased by 56% [40].

The use of tubular cellulose (TC) as yeast immobilisation carrier significantly increases the rate of alcoholic fermentation [14]. In this type of immobilisation, the cells are adhered on the carrier by physical adsorption due to electrostatic and other weak forces, as well as by entrapment of the cells into cavities on the carrier surface. The cells may grow and escape, and therefore may be in equilibrium with free cells in suspension. Apart from the ease of the immobilisation technique, which can be achieved by simple contact of a cell suspension with the carrier for a time period, TC has a number of other advantages such its availability all-year-round, suitability for extremely low temperature alcohol fermentations and production of bio-processed products with improved quality. Given that alterations in cell growth, physiology and metabolic activity may be induced by cell immobilisation (e.g. effects on the energetic metabolism activation and targeted protein expression to support it, increased substrate uptake and product yield, increased tolerance against inhibitory compounds and increased hydrolytic enzyme activities, this technology has been developed for low temperature alcohol production using immobilised yeasts and compared with the fermentation performed using free cells [33]. *Schizosaccharomyces pombe* cells entrapped in Ca alginate gel have been used under continuous fermentation conditions to evaluate the extent of malic acid degradation at different dilution rates (D, h⁻¹) and the analytical profiles of wines were assessed. Gel entrapped cells were able to cause the deacidification of wine without affecting the analytical profiles of the product. Maximum deacidification rate was obtained at 0.076 h⁻¹D while there was a reduction in activity at higher dilution rates. Whereas the deacidification activity remained constant during the observation period of 360 h at D-value of 0.055 h⁻¹[41].

7. DESIGNING OF MICROBIAL SYSTEM FOR MIXED AND CO-CULTURE FERMENTATION

Singh *et al* [42] carried out to a success simultaneous hydrolysis of starch and fermentation of released sugars to alcohol in same system of simultaneous saccharification and fermentation. *Saccharomyces cerevisiae* and *Oenococcus oeni* have been immobilized in different layers of a cellulose/starch gel composite and used to conduct simultaneous alcoholic and malolactic wine fermentations [43]. The purpose of co-immobilization of different microorganisms is with the objective of conducting different bioprocesses into the same bioreactor, simultaneously. Such combination of two types of fermentations could bring the costs of both processes down in terms of production and investment costs. However, this is natural that there is a biological competition between yeasts and lactic acid bacteria (LAB), which may cause inhibition problems. But separate entrapment of both types of microbial cells

indifferent matrices to formulate a composite biocatalyst could avoid or reduce the inhibition problems. This hypothesis was the base of our European project where Servetas *et al* [43] investigated the simultaneous alcoholic and malolactic fermentations (MLF) of grape must.

Many carriers for immobilization of yeasts and bacteria have been proposed for alcohol fermentation, including g inorganic and organic materials, mainly polysaccharides and natural products, such as cellulosic materials, cereal grains, pieces of different fruits, and starch, etc. The delignified cellulosic materials (DCM) have been used as immobilization support of yeasts and bacteria for wine making, demonstrating their favorable effect on alcoholic fermentation and MLF, and the reduction of the activation energy even at very low temperatures of wine making. DCM is a material of food-grade, cheap and abundant in nature, and the immobilization method is simple and easy. The scanning electron microscopy studies revealed that DCM contains nano-and micro-tubes facilitating extremely low temperature wine fermentation and other bioprocesses [14]. Moreover, other biomaterials, such as starch gel have also been found interesting for yeast entrapment for use in wine fermentation.

The use of immobilized bacterial cells example-*Oenococcus oeni* immobilized on DCM led to improvements in developing MLF in wine making. Therefore, the use of a biocatalyst by co-immobilization of *Saccharomyces cerevisiae* and *O. oeni* on separate layers of a composite food grade carrier can provide additional advantages in wine making [14]. The improvements in overall process were rightly anticipated as –the increased cell viabilities, resistance to low pH and ethanol concentrations, higher cell densities, simultaneous alcoholic and ML fermentation in the same bioreactor, less time for vinification at lower cost, and most importantly the improvement in the quality of final product-wine. A composite two-layer biocatalyst consisting of wheat-starch gel and tubular DCM, supported the viability and activity of immobilized yeast cells *S. cerevisiae* together with bacterial cells *O. oeni*. Such immobilized bio-catalysts could be used for wine making, to influence both productivity and quality of wine.

The simultaneous alcoholic and MLF fermentations of grape-must in the same bioreactor and effective composite biocatalyst preparation of ethanolic yeast and ML bacteria co-immobilized on Tubular DCM and starch gel have been possible. Because both types of microorganisms were located in different layers of supporting material, resulting to simultaneous performance of the two bioprocesses without any biological competition among the species. The DCM/starch gel composite biocatalyst led to improvement of wine quality, regarding the formation of volatile by-products, compared with the performance of single immobilization of organisms on separate supports.

Ethanol production by a co-culture (mixed) of *Saccharomyces diastaticus* and *Saccharomyces cerevisiae* 21 was studied using raw un-hydrolysed starch in a single-step fermentation. This was 48% higher than the yield obtained with the monoculture of *S. diastaticus*. The maximum ethanol fermentation efficiency was achieved (93% of the theoretical value) using 60 g/l starch concentration. In another co-culture fermentation with *Endomycopsis capsularis* and *S.cerevisiae* 21, maximum ethanol yield

was higher than the yield with the monoculture of *E. capsularis*. Fermentation efficiency was found lower in a two-step process using α -amylase and glucoamylase-treated starch fermentation by a single culture [44].

8. MANIPULATION OF YEASTS FOR PROCESS DEVELOPMENT

Genetic improvement has been tried to alcohol producing yeasts to introduce various qualities. Genetic engineering has

been applied to yeasts for various purposes such as introducing thermotolerance [45], but the result of research has been varying producing differing outcomes by different researchers. Genetic engineering is based on the ability to isolate specific genes from a genome and to introduce it into yeast cells through the techniques of genetic transformation. This process is based on assumption that any gene is introduced into a yeast, which is then used to start a winery fermentation; if this gene is expressed during fermentation, it will lead to the accumulation of that protein that encodes as advances for vinification and in

Table 1. Summary of Microorganisms' contribution in alcohol fermentation processing a variety of substrates.

Microorganisms *Production/Parameters	Substrate [Product]	References
Saccharomyces, non-Saccharomyces yeasts, Bacteria * Mesophilic; conventional systems	Fruits; Grapes [Red & White wines; Specialized wines]	1-3
<i>Kluyveromyces marxianus</i>	Sugarcane Molasses [Ethanol]	4-6,9
*Thermo-tolerant	High Temperature-fermentation	
<i>Saccharomyces cerevisiae</i>	Sugarcane Molasses [Ethanol]	
*Continuous production	Immobilized system	7
*Continuous production	Column Reactor	8
<i>Kluyveromyces marxianus</i>	Sugarcane Molasses [Ethanol]	
*Thermo-tolerant	Industrial-scale in Distillery ethanol production	10,11
<i>Kluyveromyces marxianus</i>	Whey, lactose, Cheese-effluent [Ethanol]	12,13
*Thermo-tolerant	Immobilized yeast cells	14, 15
	Magnetically-responsive matrices	16
	Mn ² Supplemented fermentation	17
<i>Saccharomyces cerevisiae</i>	Agricultural wastes/residues [Ethanol]	18, 19
*Batch & Fed-batch production	Sorghum, Tapioca [Ethanol]	20
Saccharomyces, non-Saccharomyces yeasts, Bacteria	Apple-must [Cider]	1-3, 21, 22
Bacteria + Yeast <i>Oenococcus</i> & <i>Saccharomyces</i> *Co-immobilized	Apple-must	23-25
Standard yeasts, Apiculated yeasts	Berries [Fruit-beverage]	3
	Plum [Fruit-beverage]	26, 27
	Papaya [Fruit-beverage]	28
	Mango [Fruit-beverage]	29
	Cagaita [Fruit-beverage]	30
Factors	Modifications	
Temperature	Thermotolerant for high temp process	4, 31, 32
	Cryotolerant for low temp process	33
Free-floating, Immobilized yeast-cells	Batch-production	36
	Continuous-production	15, 17, 37
Cells fixed on non-alginate supports - delignified cellulose	Continuous-production	40
Mixed microorganisms <i>Saccharomyces cerevisiae</i> + <i>Oenococcus oeni</i>	In layers of cellulose-starch gel-composite, Alcoholic + Malolactic fermentation	42, 43
<i>Saccharomyces diastaticus</i> + <i>S. cerevisiae</i> 21; <i>Endomycopsis capsularis</i> + <i>S.cerevisiae</i> 21	Raw starch (un-hydrolysed by amylolytic enzymes)	44
Genetic manipulations of yeasts	Improved and Varied qualities for desired products	45-47

this way to introduce the technological activity of desired application. Extensive biochemical and physiological research has been published over many years in studies of strains of *S. cerevisiae* in defined media under controlled fermentation conditions. As a result of extensive work, many of the genes associated with the metabolic generation of physico-chemical or organoleptic properties, have been cloned and sequenced which are relevant to winemaking. As an outcome of many published research, effective methods have been established for the transformation of *S. cerevisiae* and many wine strains. All available information on the use of genetic engineering can be applied to investigate the generation of improved yeasts [46].

The advanced research has defined new technologies for fermentations using molecular-biology, application of such techniques have greatly influenced the performance of yeasts in alcohol fermentation, e.g thermotolerant yeasts. The fermentation technology has been improved with the application of immobilized yeasts cells supported on natural gels of alginate and carrageenan, which is a promising development in the production of sparkling wines. The recombinant DNA technology offers the greatest potential for the improvement of wine yeasts which would necessarily bring a remarkable change in the performance of fermentation process and consequently, in the quality of final product. Yeasts with thermotolerance property have been developed for their potential use for industrial ethanol production in warm climate conditions [47].

9. CONCLUSION

The published information in literature available on this subject, provide a current information on various aspects of alcohol microbiology, and process development and production and quality refinement of product (Table 1). The conclusion of all studies is that certain process factors have to be optimised in each scenario for improved quality, if the product is to be produced using a specific starting material.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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